INFECTION AND IMMUNITY, Oct. 1971, p. 456-461

Vol. 4, No. 4
Printed in U.S.A.

Production of Staphylococcal Alpha Toxin

I. Relationship Between Cell Growth and Toxin Formation

JAMES L. DUNCAN AND GLORIA J. CHO

Department of Microbiology, Northwestern University Dental and Medical Schools, Chicago, Illinois 60611

Received for publication 2 July 1971

Alpha toxin production and its relationship to cell growth were studied in the Wood 46 strain of *Staphylococcus aureus*. Toxin first appeared in the culture in the late logarithmic stage, but at least 80% was produced during the subsequent period of slower cell growth. The toxin concentration per unit of cell mass or viable count increased continually throughout the period of toxin production and and at its maximum represented 1.6 to 2.0% of the dry weight of the cells. The possibility that alpha toxin is released as a result of cell lysis was examined by using the appearance of cellular deoxyribonucleic acid in the medium as an indicator of lysis. The results showed that no appreciable amount of lysis occurred during toxin production; at a time when almost maximum amounts of toxin were present in the culture, less than% 4 of the cells had lysed. This finding, together with the observation that less than 0.25% of the total amount of toxin in the culture could be found intracellularly, indicates that alpha toxin is released from intact cells shortly after it is synthesized.

Alpha toxin is one of an impressive number of extracellular toxins and enzymes produced by *Staphylococcus aureus*. Some of these extracellular products are thought to be involved in the pathogenesis of staphylococcal diseases, and, of this group, alpha toxin has been one of the most thoroughly studied. There appears to be nothing very unique about the amino acid composition and molecular weight (3, 5, 7, 10) of the toxin molecule, but it produces a variety of biological effects including the classical reactions of dermonecrosis, lethality, and hemolysis. Several types of cells in addition to erythrocytes are lysed by alpha toxin, and the possibility that this toxin may be an enzyme whose primary action is on the cell membrane has been proposed (2).

Although a number of studies have been concerned with the mode of action of alpha toxin, very little is known about toxin synthesis and release on the control of these processes. Raynaud et al. (17) observed that alpha toxin was liberated into the culture medium during the late logarithmic and retardation phases of cell growth, but Kapral et al. (14), investigating alpha toxin production in vivo, found that toxin was produced throughout the period of cell multiplication. Bernheimer and Schwartz (4) studied various extracellular proteins of staphylococci including alpha toxin and found substantial amounts of nucleic acid present in their 24-hr culture filtrates. This finding suggested that toxin might be liberated from the cells by autolysis, but no further evidence on this point is available.

Cellular control mechanisms must play a role in toxin production, since it is possible to obtain good growth of toxigenic staphylococci without alpha toxin production. Factors such as *p*H, CO_2, and glucose concentration of the medium are known to be important in toxin synthesis (9), and it appears that alpha toxin synthesis is not simply related to cellular protein formation (1). Because a number of questions concerning toxin production have not been resolved, we have begun an investigation into the synthesis and release of alpha toxin. An examination of toxin production in relation to the cell growth cycle is reported in this paper.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The Wood 46 strain of *S. aureus* (ATCC 10832) was used in all experiments. Stock cultures were maintained at 4 C on Brain Heart Infusion agar slants and were transferred at 6- to 8-week intervals.

The culture medium used in all experiments was Brain Heart Infusion (BBL). Flasks containing 30 ml of medium were inoculated to an optical density (OD) of 0.05 to 0.06 by resuspending the cells from 2.5-ml samples of an exponentially growing Wood 46 culture. The flasks were incubated in a reciprocating shaker water bath (90 cycles/min, 1.5-inch stroke length) at 37 C.

Toxin assay. Alpha toxin was assayed by making

dilutions of the culture supernatant fluid in phosphate-buffered saline (PBS: 0.137 M NaCl, 0.01 M phosphate; *p*H 7.0) containing 0.5% bovine serum albumin (BSA) as a stabilizing agent. An equal volume of a washed rabbit erythrocyte suspension (0.7%, v/v, in PBS) was added to the dilutions, and the mixture was incubated at 37 C for 30 min. Intact cells were removed by centrifugation, and the OD of the supernatant fluid was measured at 540 nm in a Bausch & Lomb spectrophotometer. A complete hemolysis standard was prepared by the addition of one drop of a 1% saponin solution to the same volume of erythrocytes in PBS, and the 50% hemolysis point was determined. The alpha toxin titer of the test dilutions showing approximately 50% hemolysis was calculated by using the formula (6) (OD of test dilution/OD of 50% standard) × dilution = units of hemolytic activity (HU). Staphylococcal beta toxin was assayed by a similar procedure with sheep erythrocytes. In addition, 0.001 M $MgSO_4$ was incorporated into the PBS for the beta toxin assay, and the toxin-cell suspensions were incubated for 1 hr at 37 C and then at 4 C for an additional 1 hr. Staphylococcal delta hemolysin was assayed by the same procedure described for alpha toxin by using horse erythrocytes.

Determination of cell growth. The OD of suitable dilutions of the culture was determined at 540 nm in a Bausch & Lomb spectrophotometer. Viable count determinations were made by the pour plate method in which dilutions of the culture were added to molten Brain Heart Infusion agar (BBL) at 50 C; the results were recorded as colony-forming units (CFU) per milliliter. Dry weight was determined by filtering appropriate samples of the culture on weighed, 0.45-μm membrane filters (Millipore Corp.). The bacterial cells on the filter were washed, and the filters were dried to constant weight.

DNA determination. Culture filtrates were heated to 90 C for 15 min in an equal volume of 10% trichloroacetic acid. The tubes were cooled and centrifuged, and the deoxyribonucleic acid (DNA) content of the supernatant fluid was determined by the method of Dische (8).

Radioactive compounds. Thymidine-*methyl*-^3H (6.7 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Mass. Toluene-based scintillation fluid was composed of Liquifluor (New England Nuclear Corp.) in toluene. An aqueous scintillation fluid consisted of toluene, 1,080 ml; dioxane, 1,080 ml; absolute ethanol, 650 ml; naphthalene, 250 g; 2,5-diphenyloxazole, 15 g; and 1,4-bis-2-(5-phenyloxazole)-benzene, 0.15 g. Radioactivity was measured in a Packard Tri-Carb scintillation counter.

Intracellular toxin. Three methods were used to extract intracellular toxin from the cells. In the first, the cells from growing cultures were centrifuged, washed two times in PBS containing 0.5% BSA, and resuspended in 5 ml of PBS plus BSA. The cells were disrupted in a Mickel disintegrator at 4 C for 10 min. As a result of this procedure, the viable count decreased from approximately 10^9 to 10^4 CFU/ml, and an examination of stained smears revealed very few intact cells. The lysate was filtered and assayed for toxin.

The second procedure was a modification of the procedure described by Raynaud (16). The cells were washed in PBS plus BSA and resuspended in 25 ml of a hypertonic salt solution (1 M NaCl, 0.1 M sodium citrate, 0.5% BSA). The cells were kept at 4 C for 20 min and then centrifuged, and the supernatant fluid was assayed for toxin.

A third method utilized the osmotic shock procedure shown by Heppel (13) to release a number of bacterial proteins from gram-negative cells. Cells from the culture were washed two times in 0.03 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.0, and resuspended in 0.5 M sucrose containing 0.03 M Tris and 5×10^{-3} M ethylenediaminetetraacetic acid. After shaking for 10 min, the suspension was centrifuged, and the pellet was quickly dispersed in cold 5×10^{-4} M $MgCl_2$. After shaking for 10 min in an ice bath, the cells were removed by centrifugation and the supernatant fluid was assayed for toxin after being made isotonic by the addition of NaCl.

RESULTS

Hemolysin production by strain Wood 46. The Wood 46 strain of *S. aureus* is considered to produce much more alpha toxin than most staphylococcal strains (3). When a single streak of this organism was made on rabbit blood-agar plates, a large clear zone of hemolysis surrounding the growth was observed. A very small zone of hemolysis adjacent to the steak was observed on human blood-agar plates, and the clear zone of hemolysis produced on sheep blood-agar plates after incubation at 37 C did not increase in size or change in any way when the plates were subsequently incubated at 4 C.

When 16- to 20-hr culture supernatant fluids were assayed for toxin with rabbit erythrocytes, titers of 1,600 to 2,000 HU/ml were obtained routinely. With the same supernatant fluids, less than 40 HU/ml could be detected on horse erythrocytes, and no "hot-cold" hemolysis, characteristic of staphylococcal beta toxin, could be detected on sheep erythrocytes. Based on Haque's observations on the electrophoretic identification of staphylococcal hemolysins (12), we carried out electrophoresis of 16-hr culture supernatant fluids by using a PhoroSlide electrophoresis system (Millipore Corp.). Zones of hemolysis were detected by adding a thin overlay of molten agar (Difco) at 50 C containing appropriate erythrocytes to the PhoroSlide strips which were incubated at 37 C. A single band of hemolytic activity which migrated towards the cathode was observed when the strips were overlaid with rabbit erythrocytes. An identical band was seen with sheep erythrocytes, but no additional hemolysis occurred at 4 C. No band of hemolytic activity could be detected when horse erythrocytes were used in the overlay.

The absence of "hot-cold" hemolysis and the minimal hemolytic activity against human or horse erythrocytes indicate that staphylococcal beta toxin and delta hemolysin are either absent or are present only in small amounts. Under the cultural conditions in the experiments reported here, it appears that the hemolytic activity consists almost entirely of alpha toxin.

Kinetics of alpha toxin production. The kinetics of alpha toxin production by the Wood 46 strain and their relationship to cell growth were studied. Maximum toxin production occurred when the cultures were grown in 250-ml flasks containing 30 ml of Brain Heart Infusion. Duplicate or triplicate flasks were inoculated with Wood 46 cells and, at appropriate times, samples were removed from each flask, pooled, and assayed for viable count, *p*H, and toxin. The results in Fig. 1 show that toxin production began after a 3- to 4-hr lag period and increased rapidly for 12 to 14 hr. An examination of the viable count during this time revealed that the culture grew rapidly at first, but by 5 hr the cells were entering a retarded phase of growth. Alpha toxin production began during late exponential growth, but 80% of the toxin appeared to be made during the slower phase of growth. The *p*H of the culture decreased for about 5 hr but began to increase shortly after toxin production began.

The relationship between viable count, dry weight, and OD of the culture during toxin production was examined in a similar experiment. Three flasks were inoculated as described above, except that a heavier inoculum was used to give an initial OD of about 0.6. Samples were removed at various times and assayed for cell growth and toxin production. The results in Table 1 show that, during the first 3 hr, there was almost a 10-fold increase in the number of cells and 178 HU of toxin per ml were produced. During the next 9-hr period, however, the viable count and OD approximately doubled, but the toxin titer increased by over 1,000 HU/ml. As before, it appears that most of the toxin is produced when the culture has slowed to a very low rate of growth. The viable count and OD increased proportionally during the experiment, but the dry weight increase was slower; as a result, the weight per CFU decreased throughout the growth period.

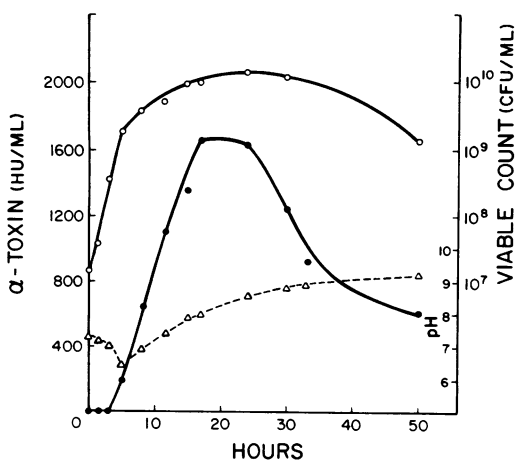

FIG. 1. *Alpha toxin production by Staphylococcus aureus strain Wood 46. Symbols:* ○, *viable count;* ●, *alpha toxin;* △, *pH.*

Loss of alpha toxin activity. The data in Fig. 1 show that the toxin concentration reached a plateau at 18 to 20 hr and began to decrease rather sharply between 24 and 30 hr. The possibility that a proteolytic enzyme(s) made late in the growth of the culture was responsible for this loss of toxin activity was examined. Two flasks were inoculated as before, and, at 17 hr, the cultures were pooled and the toxin titer was determined. A 25-ml amount of the pooled culture was placed into each of the two flasks, one of which also received chloramphenicol (100 μg/ml). The flasks were incubated, and samples were taken at various times and assayed for toxin. We expected the addition of chloramphenicol at this late time to have little or no effect on the total amount of toxin produced, except perhaps to protect the toxin which had already accumulated in the medium. The results in Fig. 2, however, show that toxin activity was lost more rapidly in the chloramphenicol-treated culture. In a similar experiment, 16-hr cultures were pooled and divided into two 25-ml portions. The cells were removed from one portion by centrifugation, and the supernatant fluid alone was added to a 250-ml flask. The flasks were incubated as above and sampled for toxin. The results showed that the loss of toxin activity occurred much more rapidly in the flask containing supernatant fluid alone; the kinetics were almost identical to those shown in Fig. 2.

TABLE 1. *Optical density, viable count, and dry weight of Staphylococcus aureus cultures during alpha toxin production*

Time (hr)	Optical density (540 nm)	Dry weight (mg/ml)	Viable count (CFU/ml)	Toxin (HU/ml)
0	0.59	0.43	5.0×10^{8}	ND[a]
1.5	2.97	1.43	2.3×10^{9}	ND
3.0	5.45	2.50	4.8×10^{9}	178
8.0	8.10	3.70	7.7×10^{9}	888
12.0	9.21	4.10	10.5×10^{9}	1,277
25.0	9.59	4.20		1,113

[a] Not detectable.

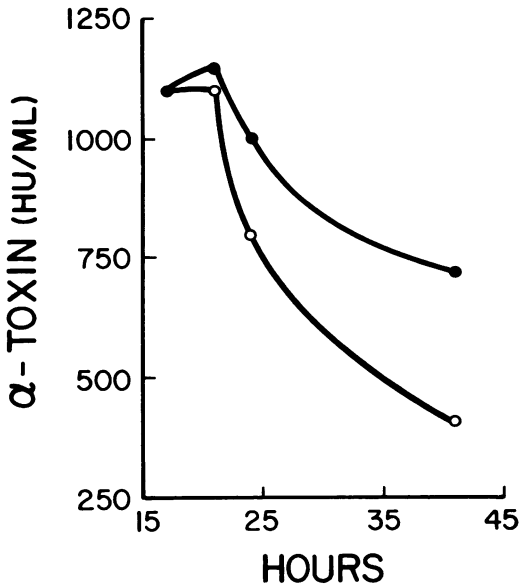

FIG. 2. *Effect of chloramphenicol on the loss of alpha toxin activity. Chloramphenicol (100 μg/ml) was added to one culture after 17 hr of growth. Symbols: ○, chloramphenicol-treated; ●, control.*

Finally, the possibility that an inhibitor of toxin activity accumulated in the medium was tested by mixing appropriate amounts of supernatant fluid from 16- and 42-hr cultures. The tubes were incubated for 1 hr at 37 C, and the toxin concentration of each mixture was determined. As can be seen in Table 2, there was no loss of toxin activity when the toxin preparations were incubated together; in fact, the toxin titers of the mixtures were slightly greater than additive. Longer incubation times did not alter the results, and it appears that the loss of toxin activity from older cultures is not due to the presence of an inactivating substance in the medium.

Toxin production and cell lysis. Based on OD 260:280 absorption ratios and ribose determinations, Bernheimer and Schwartz concluded that appreciable amounts of ribonucleic acid (RNA) were present in culture supernatant fluids of several staphylococcal strains (4). Alpha toxin was one of the extracellular products examined in their study, raising the possibility that the release of toxin occurs as a result of cell lysis. We examined this possibility by following the appearance of cellular deoxyribonucleic acid (DNA) in the culture medium during toxin production.

A flask was inoculated, and, at various times, samples were removed and centrifuged. The supernatant fluid was filtered through a 0.45-μm membrane filter (Millipore Corp.), and the filtrate was assayed for toxin and deoxyribose by using the diphenylamine reagent (8). The results in Table 3 show that the diphenylamine-positive material present in Brain Heart Infusion decreased during the period of rapid cell growth so that by 4 hr 72 μg/ml was present. During the next 4-hr period, the toxin titer increased by almost 700 HU/ml, whereas the diphenylamine-reacting material increased by only 2 μg/ml; even after 26 hr, the increase was only 6 μg/ml. The high background of diphenylamine-positive material in Brain Heart Infusion makes a precise interpretation difficult, but this experiment does indicate that gross lysis of the culture does not occur during toxin production.

To examine the relationship between cell lysis and toxin production more critically, Wood 46 cells were inoculated into Brain Heart Infusion containing 16.7 μCi of ^{3}H-thymidine per ml. After a 4-hr growth period, the cells were washed two times with ice cold Brain Heart Infusion and resuspended in 25-ml of Brain Heart Infusion. Samples (1.5 ml) were taken at various times, and appropriate amounts (0.05 or 0.01 ml) were added to 1 ml of PBS. The cells in this suspension were collected on membrane filters which were dried and placed in scintillation fluid. The remainder of the sample was centrifuged, and the supernatant fluid was filtered through a membrane filter

TABLE 2. *Incubation of 16-hr toxin with 42-hr toxin*

Tube	Culture supernatant fluid		pH	Toxin	
	16 Hr (ml)	52 Hr (ml)		Expected (HU/ml)	Experimental (HU/ml)
1	2.0	0	7.9		1,520
2	0	2.0	8.9		665
3	1.8	0.2	8.0	1,437	1,535
4	0.2	1.8	8.9	750	860
5	1.0	1.0	8.5	1,092	1,130

TABLE 3. *Release of diphenylamine-reacting material during alpha toxin production*

Time (hr)	Optical density (540 nm)	Diphenylamine color as deoxyribose (μg/ml)	Toxin (HU/ml)
0	0.108	137	ND[a]
4	5.090	72	91
8	8.640	74	783
26	10.460	78	1,152
50	9.380	83	324
74	9.380	93	144

[a] Not detectable.

TABLE 4. *Release of ^{3}H-thymidine from labeled cells during toxin production*

Time (hr)	Optical density (540 nm)	^{3}H in supernatant fluid (counts per min per ml)	^{3}H in cellular material (counts per min per ml)	Toxin (HU/ml)
0	5.36	440	20,560	ND[a]
2.0	10.58	440	20,440	156
3.5	11.70	580	21,880	301
6.0	13.16	840	20,800	450
8.0	13.16	940	19,900	974
9.5	13.56	1,160	19,820	1,239
20.0	13.98	4,360	19,380	1,090

[a] Not detectable.

(Millipore Corp.). Toxin was assayed, and the amount of radioactivity in the filtrate was determined by adding 0.05-ml amounts to an aqueous scintillation fluid (Table 4). A background of 440 counts/min in the supernatant fluid was present at "0" time. The amount of radioactivity in the supernatant fluid increased only slightly during toxin production, and, by 9.5 hr, when over 1,200 HU of toxin per ml had been made, 720 counts per min per ml above the background level were present in the supernatant fluid; this represents less than 4% of the radioactivity present in the cells.

Intracellular alpha toxin. The possibility that toxin accumulated within the cell during the "lag" period (Fig. 1) or at other times during toxin production was studied. Wood 46 cultures were incubated for 4, 8, or 16 hr, at which time the cells were removed by centrifugation. The supernatant fluid was assayed for extracellular toxin, and the cells were washed and disrupted in a Mickle disintegrator or extracted by one of the procedures described above. At no time did the intracellular toxin released by these procedures represent more than 0.25% of the total toxin present in the culture. The cell-associated toxin (toxin in wash plus intracellular toxin) was only 1 to 2% of the total toxin in the culture.

DISCUSSION

The experiments described in this report show that the growth curve of *S. aureus* strain Wood 46 consists of an initial period of logarithmic growth followed by an extended period of relatively slow growth. No alpha toxin can be detected early, and it is only in the latter part of the exponential growth period that toxin production begins; however, 80 to 90% of the total toxin produced by the culture is made during the subsequent period of slower cell growth. Thus, it appears that toxin production does not simply parallel growth of the culture. This observation is confirmed when the data in Table 1 are calculated in terms of HU of toxin produced per unit of cell mass or viable count; toxin synthesis per milligram (dry weight), OD at 540 nm, or CFU increases continually during the period of toxin production.

These results appear to be in complete agreement with those Mangalo et al. (15) and Raynaud et al. (17), who found that alpha toxin first appeared in their Wood 46 cultures after a 3-hr lag period at which time the cells were entering a phase of retarded growth. Kapral et al. (14) studied alpha toxin synthesis by staphylococci in dialysis sacs which had been implanted in the peritoneal cavity of mice. They found that toxin production closely paralleled growth of the organisms and that after the third generation toxin synthesis per generation remained constant. The differences in their results and the observations reported here might be due to the different staphylococcal strains used or may reflect actual differences in toxin production during in vitro and in vivo growth. However, Gladstone and Glencross (11) studied alpha toxin synthesis in vivo and found that toxin production lagged behind cell growth, reaching maximum titers some 6 to 8 hr after maximum growth. Their finding would appear to be more compatible with our results.

Maximum toxin concentrations are found in the culture at 15 to 20 hr. Based on the number of HU per milligram of purified toxin protein (5), the concentration of alpha toxin during this period represents 1.6 to 2.0% of the dry weight of the cell; Bernheimer and Schwartz (5) found a similar ratio. The toxin titer remains stationary at this high level for several hours and then begins to decrease. When chloramphenicol was added to a culture at 17 hr, the toxin titer decreased much more rapidly than in control cultures (Fig. 2). The same phenomenon was observed when cells were removed from a culture at 16 hr, suggesting that small amounts of toxin may continue to be produced very late in the culture. The stationary phase of toxin synthesis may represent a condition in which toxin in the culture is being produced at about the same rate at which it is being inactivated. We could find no evidence that the subsequent decrease in toxin titer was due to the presence of a substrate in the culture medium which inactivated toxin. The most plausible explanation is that toxin is inactivated simply as a result of surface denaturation caused by continued shaking at 37 C.

There seems to be no doubt that the cells are growing, albeit slowly, during the time that toxin is produced, and the possibility that toxin is liberated into the medium as a result of cell lysis

does not seem to be the case. Based on the appearance of cellular DNA (or DNA precursors) in the medium, the amount of cell lysis which occurs during toxin production appears to be insignificant. When cellular DNA was labeled with ^{3}H-thymidine and the appearance of radioactivity in the medium was followed, only slight increases above the background level could be detected during the period of toxin production. At the time when nearly maximal toxin concentrations were reached in the medium, less than 4% of the cells had lysed. Also related to the question of toxin release is the finding that less than 0.25% of the total toxin concentration in the culture can be found intracellularly. Mangalo et al. (15) found no intracellular toxin when their cultures were disrupted by ultrasonic treatment or allowed to lyse in the presence of penicillin. The possibility that the procedures used to release intracellular toxin may not be effective must be kept in mind, but it seems safe to conclude that alpha toxin does not accumulate within the cells to any appreciable extent. Furthermore, the addition of chloramphenicol (100 μg/ml) to cultures during their most active period of toxin synthesis inhibits further toxin production within a matter of minutes (*unpublished data*). Taken together, these results strongly suggest that alpha toxin is released from intact cells very shortly after it is synthesized.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant AI 08793 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Arbuthnott, J. P. 1970. Staphylococcal α-toxin, p. 189-236. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
2. Bernheimer, A. W. 1968. Cytolytic toxins of bacterial origin. Science **159**:847-851.
3. Bernheimer, A. W. 1970. Cytolytic toxins of bacteria, p. 183-212. *In* S. J. Ajl, S. Kadis, and T. C. Montie (ed.), Microbial toxins, vol. 1. Academic Press Inc., New York.
4. Bernheimer, A. W., and L. L. Schwartz. 1961. Extracellular proteins of staphylococci. Proc. Soc. Exp. Biol. Med. **106**: 776-780.
5. Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**:455-468.
6. Cooper, L. Z., M. A. Madoff, and L. Weinstein. 1964. Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. I. Kinetics of the lytic reaction. J. Bacteriol. **87**:127-135.
7. Coulter, J. R. 1966. Production, purification, and composition of staphylococcal α toxin. J. Bacteriol. **92**:1655-1662.
8. Dische, Z. 1955. Color reactions of nucleic acid components, p. 285-305. *In* E. Chargaff and J. N. Davidson (ed.), The nucleic acids, vol. 1. Academic Press Inc., New York.
9. Elek, S. D. 1959. Staphylococcus pyogenes and its relation to disease. E. & S. Livingstone, Edinburgh.
10. Forlani, L., A. W. Bernheimer, and E. Chiancone. 1971. Ultracentrifugal analysis of staphylococcal alpha toxin. J. Bacteriol. **106**:138-142.
11. Gladstone, G. P., and E. J. G. Glencross. 1960. Growth and toxin production of staphylococci in cellophane sacs in vivo. Brit. J. Exp. Pathol. **41**:313-333.
12. Haque, R. 1967. Identification of staphylococcal hemolysins by an electrophoretic localization technique. J. Bacteriol. **93**:525-530.
13. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science **156**:1451-1455.
14. Kapral, F. A., A. M. Keogh, and J. H. Taubler. 1965. The nature of alpha toxin production by *Staphylococcus aureus* grown *in vivo*. Proc. Soc. Exp. Biol. Med. **119**:74-77.
15. Mangalo, R., J. Pillet, and M. Raynaud. 1954. Études sur la toxine α de *Staphylococcus pyogenes*. Rapports entre croissance et toxinogénése. C. R. Acad. Sci. **238**:1931-1933.
16. Raynaud, M. 1947. Extraction de la toxine de *Clostridium sordelli* á partir des corps bactériens. C. R. Acad. Sci. **225**:543-544.
17. Raynaud, M., A. Turpin, R. Mangalo, and B. Bizzini. 1955. Croissance et toxinogenèse. Ann. Inst. Pasteur **88**:24-43.